Supplemental Data to Lien et al. “Dengue virus and antiplatelet autoantibody synergistically induce haemorrhage through Nlrp3-inflammasome and FcγRIII” (Thromb Haemost 2015; 113.5)

Suppl. Figure 1: DENV-challenge induces hypercoagulation in mice. Experiment outline (A) of activated partial thromboplastin time (aPTT) (B) and prothrombin time (PT) (C), which analysed the intrinsic and extrinsic coagulant pathways, respectively, were performed 0, 2, 8, 24 hr after DENV (3 x 10^5 PFU/mouse; PL046) challenges. The percentage of total leukocytes that are of tissue factor^+, were also analysed by 24 hr after the diluent (vehicle) and DENV challenges (D). Data are represented as mean +/- SD. ** P < 0.01 significantly exacerbated hypercoagulation versus respective 0 hr or vehicle groups. n = 6 (three experiments with two replicates).
Suppl. Figure 2: Quantification of double-hit induced haemorrhage.

Traditional measurements for the haemorrhagic lesions in local Shwartzman reaction were empirically examined and scored on a scale of 0 to 4 (1, 2). To obtain objectively and scientifically measureable data, experiment outlines of photographing of mouse skin (A) and the data processing (B, C) were established. The images were taken in red-green-blue (RGB) colour mode at a consistent condition (sample-to-camera distance 7 cm, illumination intensity 200 lux) (A) without further brightness and/or contrast adjustments (B, C). Because red and green are complementary colours, the haemorrhage score (a.u.; relative levels of red intensity and area) of a skin-sample could be generally obtained using Image J-measured mean intensity of the red channel to subtract with the mean intensity of the green channel of the same image (B, C). The haemorrhage scores of representative images in panel D were showed (E).
Suppl. Figure 3: Characterisation of DHF-related manifestations in two-hit mouse model. The experimental outline was illustrated (A). Mice were infected with (DENV groups) or without (vehicle) DENV (three dosages: $3 \times 10^5$, $1.2 \times 10^6$, $2.4 \times 10^6$ PFU/mouse; 1st-injection; PL046) for the first 24 hours and then were challenged by various antibody treatments (2nd-injections; control Ig, anti-CD41 Ig, anti-NS1 Ig) (B-E). Reduced levels of platelet counts (B) and plasma protein concentration (C), and the elevated aspartate transaminase (AST) levels (D) were measured. The mortality (E) was also recorded. Results showed as mean +/- SD. (B-D) Red asterisks * $P < 0.05$, **$P < 0.01$ indicate significantly changes vs DENV + control Ig (Ctrl Ig) groups; # $P < 0.05$, ##$P < 0.01$ indicate significantly changes vs vehicle groups. (E) The $P$ value showed was compared to respective DENV + control Ig groups; the labels DENV*1, DENV*4 and DENV*8 indicate DHF-viral-load dosages $3 \times 10^5$, $1.2 \times 10^6$, $2.4 \times 10^6$ PFU/mouse, respectively. (B-D) n = 6 (three independent experiments with two replicates); (E) Anti-CD41 Ig, n = 9; anti-NS1 Ig, n = 8; other groups n = 6. All survived mice were under monitor until 2 month, and no further casualty was observed within this period of time (21 day-2 month).
Suppl. Figure 4: Two-hit induced IL-10 and coagulant disturbances in wild type C57Bl/6J mice. Induction of circulating IL-10, anticoagulant deficiency (e.g. antithrombin III and protein C) and coagulopathy has been reported in various DHF cases (3-6). Simplified changes of clinical parameters during DHF (A), and the experimental outline (B) were illustrated. Mice were infected with (panels 8–14) or without (panels 1–7) DENV (1st-injection; PL046) for the first 24 hours and then were challenged by various treatments (2nd-injections) (B). Changes of circulating levels of IL-10 (C), anticoagulants protein C and antithrombin III (D), and D-dimer (E), were recorded. Results showed as mean +/- SD. Red asterisks * P < 0.05, **P < 0.01 indicate significantly worse changes vs DENV + control Ig-1 groups; #P < 0.05, ##P < 0.01 indicate significantly worse changes vs vehicle groups. Vehicle, DENV, DENV + anti-CD41 Ig, n = 20; anti-CD41 Ig, DENV + control Ig, DENV + anti-NS1 Ig, n = 15; DENV + anti-envelope Ig, n = 10; other groups n = 6. Control Ig-1: rabbit preimmune control IgG; control Ig-2: rat isotype control Ig.
Suppl. Figure 5: The viral load. Circulating virus titers were detected 5, 15, 30, 60, 120 minutes (A) and 24, 48, 72, 96 hours (B) after subcutaneously injection of DENV (3 × 10^5 PFU/mouse; PL046). Blood samples were collected from the retro-orbital venous plexus of mice. ** P < 0.01 versus 5 minute groups (n = 3).
Suppl. Figure 6: Two-hit-induced pathogenesis (DENV2 NGC). Changes in clinical parameters during DHF (A) and the experimental outline (B) are shown. (C–F) Mice with (panels 7–12) or without (panels 1–6) DENV (3 x 10^5 PFU/mouse; 1st-injection; DENV2 NGC) were challenged by various treatments (2nd-injections). The changes in (C) platelet counts, (D) haemorrhagic lesions, (E) haemorrhage score, and (F) proinflammatory cytokines were then recorded. Control Ig: rabbit preimmune control IgG. Results are shown as mean +/- SD. Red asterisks * P < 0.05, **P < 0.01 indicate significantly worse conditions vs DENV + control Ig groups; #P < 0.05, ##P < 0.01 vs vehicle groups. n = 6 (3 independent experiments with 2 replicates).
Suppl. Figure 7: Two-hit-induced pathogenesis (DENV4 H241). Changes in clinical parameters during DHF (A) and the experimental outline (B) are shown. (C–F) Mice with (panels 7–12) or without (panels 1–6) DENV (3 x 10^5 PFU/mouse; 1st-injection; DENV4 H241) were challenged by various treatments (2nd-injections). The changes in (C) platelet counts, (D) haemorrhagic lesions, (E) haemorrhage score, and (F) proinflammatory cytokines were then recorded. Control Ig: rabbit preimmune control IgG. Results are shown as mean +/- SD. Red asterisks * P < 0.05, **P < 0.01 indicate significantly worse conditions vs DENV + control Ig groups; #P < 0.05, ##P < 0.01 vs vehicle groups. n = 6 (3 independent experiments with 2 replicates).
Suppl. Figure 8: Involvement of the TLR3 pathway. Experimental outline (A) and the changes of mouse platelet counts (B), haemorrhage lesions (C-D), proinflammatory cytokines (E), IL-10 (F), circulating anticoagulants protein C and antithrombin III (G), and D-dimer (H), were showed. *Tlr3<sup>−/−</sup> and *Tlr4<sup>−/−</sup> mutants were compared with wild type (WT) mice after first injections of DENV (PL046) and then challenges of anti-CD41 (α-CD41) Ig for 24 hours (B-H) (n = 6). Data are represented as mean +/- SD. * P < 0.05, ** P < 0.01 significant less responsiveness versus corresponding wild-type groups. n = 6 (three experiments with 2 replicates).
Suppl. Figure 9: Nlrp3 inflammasome involves the coagulant disturbance. Experimental outline (A) and the changes of mouse circulating IL-10 (B, E) anticoagulants protein C and antithrombin III (C, F), and D-dimer (D, G), were showed. (B-D) Treatments of caspase inhibitor Z-VAD ameliorated abnormal coagulant changes of two-hit treated wild type (WT) mice (panels 3, 5 vs 2, 4). (E-G) The coagulant disturbance of Nlrp3\(^{-/-}\) (panels 4–6) and Casp1\(^{-/-}\) (panels 7–9) mutants were compared to that of wild type (panels 1–3) mice after DENV (PL046) and antibody two-hit treatments. (n = 6; D–E panels 6, 9, n = 4). Data are represented as mean +/- SD. \#P < 0.05, ##P < 0.01 indicate significantly better conditions vs DENV + anti-CD41 (α-CD41)/anti-NS1 (α-NS1) Ig groups (B-D). Red asterisks *P < 0.05, **P < 0.01 vs DENV + control (Ctrl) Ig groups; black asterisks *P < 0.05, **P < 0.01 vs respective wild type groups (E-G).
Suppl. Figure 10: Induction of FcyRIII⁺ leukocyte population after double-hit challenges. Experiment outline is illustrated (A). Because anti-mouse FcyRIIB Ig is not available, to investigate the leukocytic expression of FcyRIIB and FcyRIII, the percentage of FcyRIIB/RIII⁺ (antibody recognizes both FcyRIIB and FcyRIII) and FcyRIII⁺ populations of total CD14⁺ leukocytes from wild type (B) and Fcgr2b⁻/⁻ mice (C) were measured after the double-hit treatments. * P < 0.05, ** P < 0.01 significant elevation versus respective vehicle groups. ** P < 0.01 significantly less responsiveness versus respective DENV (PL046) + anti-CD41 Ig groups. The staining patterns are similar between FcyRIIB/RIII⁺ and FcyRIII⁺ populations in wild type and Fcgr2b⁻/⁻ mice, suggesting that the changes primarily indicate the FcyRIII expression. In addition, treatments of IVIg are able to ameliorate such induction on FcyRIII⁺ leukocytes. n = 6 (three experiments with 2 replicates). Data are represented as mean +/- SD.
Suppl. Figure 11: FcγRIII pathway involves the coagulant disturbance.
Experimental outline (A) and the changes of mouse circulating IL-10 (B, E) anticoagulants protein C and antithrombin III (C, F), and D-dimer (D, G), were showed. (B–D) The IVlg treatments ameliorated abnormal coagulant changes of two-hit challenged wild type (WT) mice (panels 3, 5 vs 2, 4). (E-G) The coagulant disturbance of Fcgr2b−/− (panels 4–6) and Fcgr3−/− (panels 7-9) mutants were compared to that of wild type (panels 1-3) mice after two-hit challenges. (n = 6; B–C panel 5, n = 4). Data are represented as mean +/- SD. #P < 0.05, ##P < 0.01 indicate significantly better conditions vs DENV (PL046) + anti-CD41 (α-CD41)/anti-NS1 (α-NS1) Ig groups. Red asterisks *P < 0.05, **P < 0.01 vs DENV + Ctrl Ig groups; black asterisks *P < 0.05, **P < 0.01 vs respective wild type groups.
Suppl. Figure 12: IL-1β and TNF-α pathways involve the coagulant disturbance. Experimental outline (A) and the changes of mouse circulating IL-10 (B) anticoagulants protein C and antithrombin III (C), and D-dimer (D), were showed. (B-D) Treatments of IL-1RA or etanercept (IL-1 and TNF-α antagonist, respectively) ameliorated abnormal IL-10 induction (B) and coagulant changes (C, D) of two-hit challenged wild type (WT) mice. (n = 6; B–C panels 3, 6, 7, n = 4). Data are represented as mean +/- SD. #P < 0.05, ##P < 0.01, was significantly ameliorated condition as compared to the respective DENV (PL046) + control (Ctrl) Ig groups.
Suppl. Figure 13: Two-hit model for infant DHF. (A) Mouse models for classic DHF (a-1, for children and adults) and infant DHF (a-2), and (B) the hypothetical timing and parameter changes of circulating DENV-elicted Ig (first-hit events [a]), DENV (second-hit events [b]) in DHF infants are illustrated. Notably, the first hit and the second hit were theoretically reversed in the classic DHF versus the infant...
DHF (a-1 vs a-2). In the classic DHF, patients received second DENV infection and then induced DENV-elicited Ig (a-1). By contrast, in infant DHF, DENV-elicited Ig was maternally derived before the primary DENV infection (a-2; b [a1-2, b1-2]). When DENV (the second hit) reached to a pathogenic threshold (b [b2]), both hits are thus sufficient to initiate DHF (B [c2] vs [c1, c3]). The changes of (C) platelet counts, (D) haemorrhage lesions, (E) haemorrhage score, (F) proinflammatory cytokine (IL-1β, TNF-α), (G) IL-10, (H) anticoagulants protein C, antithrombin III, and (I) D-dimer of each mouse groups were recorded 24-hour post the second hit challenges (n = 6). DENV2 PL046 was used. Data are represented as mean +/- SD. * P < 0.05, ** P < 0.01, compared with respective anti-platelet (anti-CD41) Ig (first-hit only) groups.
Suppl. Table 1. Ameliorative effects of therapeutic agents in double-hit challenges. Therapeutic agents IVlg, Z-VAD-FMK, IL-1RA, etanercept were treated 10 min after the first-hit (treated after 1st hit) or second-hit (treated after 2nd hit) challenges of wild type C57Bl/6J mice. Parameters including haemorrhage score, IL-1β, TNF-α, protein C, antithrombin III and D-dimer were analysed. * P < 0.05, ** P < 0.01 significantly ameliorated condition versus respective DENV (PL046) + anti-CD41 Ig groups. Colour -labeled panels highlight those treatments with a significant better effect versus respective treatments at another time point (treatments after 1st hit versus 2nd hit; † P < 0.05, †† P < 0.01). n = 4 in all IL1-RA treated groups; others n = 6 (two or three experiments with 2 replicates). Data are represented as mean +/- SD.
Suppl. References


